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Characterization of the minor groove environment in a drug–DNA complex: Bisbenzimide bound to the poly[d(AT)]·poly[d(AT)]duplex

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ABSTRACT **We compare the fluorescence properties of bisbenzimide (also known as Hoechst 33258) bound to the minor groove of the poly[d(AT)]·poly[d(AT)] duplex with the corresponding fluorescence properties of bisbenzimide dissolved in neat organic solvents and mixed organic/aqueous solvents. Based on these comparisons, we conclude that the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex is quite nonpolar and exhibits a local dielectric constant of ≈20 D. We discuss how this insight influences our understanding of the molecular forces that dictate and control the binding affinities and specificities of minor groove-directed DNA binding ligands.**

The molecular forces that dictate and control the affinities and the specificities of DNA binding ligands are modulated by the microenvironments in which they are expressed. For example, salt bridges and hydrogen bonds are more favorable in low dielectric environments, while hydrophobic forces generally are enhanced in high dielectric environments. Consequently, the microenvironments within drug–DNA complexes must be characterized before one can define the relative contributions that specific molecular interactions make to the DNA binding of a particular class of ligands.

A DNA microenvironment of particular interest is the minor groove of B-form duplexes, since an important class of nonintercalating ligands binds to this DNA domain. These nonintercalating ligands have been studied as models for protein–DNA and drug–DNA recognition patterns and as sequence-specific delivery systems for affinity cleaving reagents. However, before one can assess the relative contribution that each ligand–DNA interaction makes to the binding affinity and specificity of a nonintercalating ligand, the local environment within the minor groove of the drug–DNA complex must be characterized. To this end, we have used bisbenzimide as a probe of the microenvironment within the minor groove of a drug–DNA complex. This ligand is ideally suited for this purpose since it is highly fluorescent and it selectively binds to AT regions in the minor groove of B-form DNA with a high binding constant, thereby precluding complications from the fluorescence of the unbound state.

In this paper, we compare the fluorescence properties of bisbenzimide in its DNA-bound state with the corresponding properties of the free ligand in neat organic solvents and mixed organic/aqueous solvents. Based on these comparisons, we are able to characterize qualitatively the minor groove microenvironment in which the bisbenzimide–DNA interactions are expressed.

EXPERIMENTAL PROCEDURES

Materials. Bisbenzimide was obtained from Aldrich Chemical and was used without further purification. The structure of this drug is shown in Fig. 1. This drug is also called Hoechst 33258. The chemical name is 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride pentahydrate. A stock solution of bisbenzimide was prepared by dissolving it in a 10 mM phosphate buffer (pH 5). This pH was used to enhance the aqueous solubility of the drug. The concentration of the stock solution was determined spectroscopically by using a bisbenzimide extinction coefficient of 4.1×10^4 dm^3·mol^{-1}·cm^{-1} at 339 nm (1). The organic solvents used in this work (1,4-dioxane and short-chain aliphatic alcohols) were obtained from Aldrich Chemical and had purity ratings of at least 99.5%. Each organic/aqueous solvent system was prepared by mixing distilled water with the appropriate volume percent of 1,4-dioxane. This procedure produced mixed solvents with pH values of about 5.

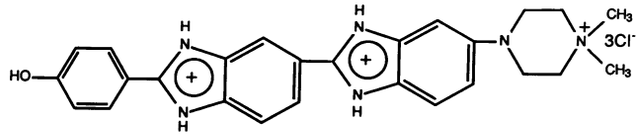

FIG. 1. The structure of bisbenzimide.

The dielectric constant (ε) and the refractive index (n) of each solvent system used are listed in Tables 1 and 2. These data can be combined to derive the orientation polarity (f) of each solvent according to the equation

$$f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}.$$

The orientation polarity, f, provides a useful means of characterizing the bulk properties of each solvent system (4).

The self-complementary alternating DNA copolymer poly[d(AT)] was purchased from Pharmacia. Aqueous solutions of the polynucleotide were made with 10 mM phosphate buffer (pH 7.0). Solutions containing the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex were prepared with a drug/DNA ratio of 1 bisbenzimide/100 phosphates.

Methods. A Perkin-Elmer MPF-66 spectrometer was used to measure the corrected excitation and emission spectra for bisbenzimide in each solvent system and for bisbenzimide complexed with poly[d(AT)]·poly[d(AT)]. Emission and excitation wavelength maxima were defined by determining the wavelength of maximum intensity after smoothing each spectrum by using a quadratic polynomial. The difference between the wave number of maximum excitation and emission is called the Stokes' shift ($\Delta\mu$). We calculated Stokes' shifts for bisbenzimide in each solvent system and for bisbenzimide complexed with poly[d(AT)]·poly[d(AT)]. The resulting Stokes' shifts then were plotted against the orientation polarity, f, of each solvent system. Such plots are linear if the influence of solvent on our fluorescence observables is dominated by general rather than specific solvent effects (4, 5).

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Table 1. Physical properties of neat organic solvents and the Stokes' shifts of bisbenzimide in each solvent

Neat organic solvent	ε*	n	f	λ_{ex}, nm	λ_{em}, nm	$\Delta\mu$, cm^{-1}
MeOH	32.63	1.329	0.308	347	498	8740
EtOH	24.30	1.361	0.288	347	485	8200
1-PrOH	20.1	1.384	0.274	347	479	7940
1-BuOH	17.1	1.398	0.263	347	471	7590
1-PeOH	13.9	1.409	0.250	348	463	7140
Poly[d(AT)]·poly[d(AT)]				355	489	7720

λ_{ex}, wavelength that corresponds to the maximum in the excitation (ex) spectrum of bisbenzimide in each solvent; λ_{em}, wavelength that corresponds to the maximum in the emission (em) spectrum of bisbenzimide in each solvent.
*Data from ref. 2.

RESULTS AND DISCUSSION

The Influence of Solvent on Bisbenzimide Fluorescence. To evaluate the influence of solvent environment on the fluorescence of free bisbenzimide in solution, we measured the excitation and emission spectra of the drug in five neat organic solvents and in a series of dioxane/water mixed solvents. The results obtained from these measurements are described below.

Neat organic solvents. Fig. 2 (*Upper*) shows the corrected excitation and emission spectra obtained for bisbenzimide in 10 mM phosphate buffer (pH 7.0) and in five different neat organic solvents. The Stokes' shifts associated with these spectra are listed in the final column of Table 1. Note that the magnitude of the Stokes' shifts varies with the nature of the organic solvent. If this solvent-dependent fluorescence behavior primarily reflects a general rather than a specific solvent effect, then a plot of the Stokes' shift versus the orientation polarity of the solvent should yield a straight line. The open squares in Fig. 3 show that we do in fact observe such a linear relationship. Consequently, we conclude that general rather than specific solvent effects are operative in the neat solvent systems studied here. It should be noted that each data point in Fig. 3 is labeled with a designation that reflects the solvent composition. Specifically, C1 corresponds to methanol, C2 to ethanol, C3 to 1-propanol, C4 to 1-butanol, and C5 to 1-pentanol.

Mixed solvents. We used dioxane/water mixed solvent systems to expand the range of solvent environments produced by the five neat organic solvents noted above. Fig. 2 (*Lower*) shows the corrected excitation and emission spectra of bisbenzimide in these mixed solvents as well as in distilled water. The three labels shown correspond to the volume percent of dioxane present in the mixed solvent system. The Stokes' shift data derived from these spectra are listed in the last column of Table 2. Paralleling the treatment used above for the neat organic solvents, we plotted these Stokes' shifts against the orientation polarities of the mixed solvent systems. The data are presented in Fig. 3 (solid diamonds) with the initial, mid-, and end points labeled by a number that corresponds to the volume percent of dioxane in the mixed solvent system. Inspection of these data show that for the mixed solvents we observe a linear relationship that essentially is superimposable on the neat organic solvent line (open squares). Thus, despite the potential for selective solvation effects in organic/aqueous mixed solvent systems, bisbenzimide exhibits fluorescence behavior that is characteristic of a general rather than a specific solvent effect.

Table 2. Physical properties of dioxane/water mixed solvents and the Stokes' shifts of bisbenzimide in each solvent

1,4-Dioxane*	ε†	n	f	λ_{ex}, nm	λ_{em}, nm	$\Delta\mu$, cm^{-1}
0	78.5	1.333	0.320	345	515	9570
5	72.8	1.338	0.317	347	512	9290
10	67.0	1.343	0.314	347	510	9210
15	63.3	1.349	0.312	347	508	9130
20	58.2	1.354	0.309	348	506	8970
25	54.2	1.359	0.306	348	503	8860
30	50.4	1.364	0.303	348	500	8740
35	45.8	1.369	0.300	348	497	8620
40	41.3	1.374	0.296	348	492	8410
45	37.3	1.379	0.292	348	490	8330
50	32.7	1.383	0.288	348	486	8160
55	28.2	1.389	0.283	348	481	7950
60	24.0	1.392	0.277	348	478	7820
65	20.0	1.397	0.270	348	475	7680
Poly[d(AT)]·poly[d(AT)]				355	489	7720

*Vol % of 1,4-dioxane in mixed solvent system with water.
†Data from ref. 3.

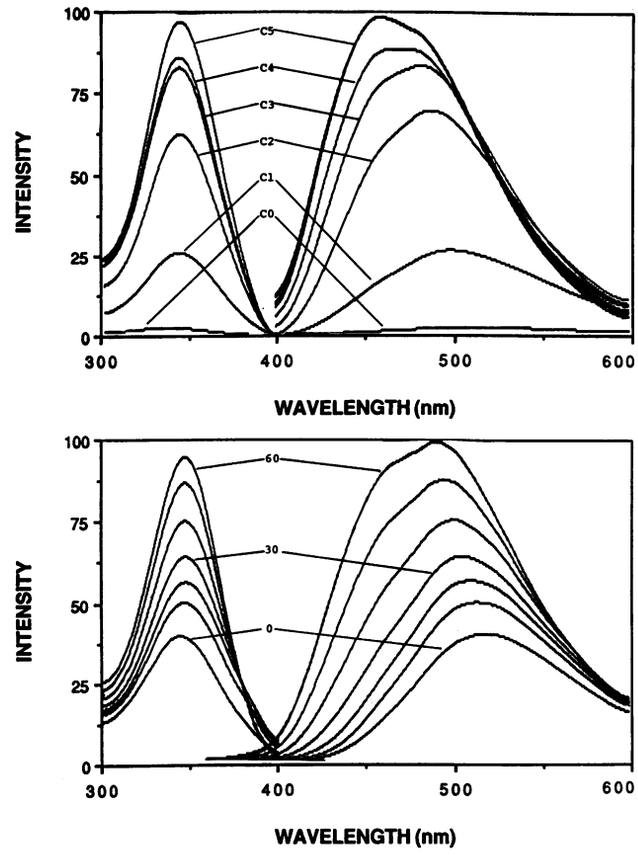

FIG. 2. (*Upper*) The corrected emission spectra (family on the right) and excitation spectra (family on the left) of bisbenzimide in five neat organic solvents and in 10 mM phosphate buffer (pH 7.0). The bisbenzimide spectra were obtained in 10 mM phosphate buffer at pH 7.0 (C0), methanol (C1), ethanol (C2), 1-propanol (C3), 1-butanol (C4), and 1-pentanol (C5). Bisbenzimide concentration, 4×10^{-7} M; excitation slit, 5.0 nm; emission slit, 2.0 nm. (*Lower*) The corrected emission spectra (family on the right) and excitation spectra (family on the left) of bisbenzimide in 1,4-dioxane/water mixed solvents. From top to bottom, the bisbenzimide spectra were obtained in 60, 50, 40, 30, 20, 10, and 0 vol % 1,4-dioxane in dioxane/water mixed solvents. Bisbenzimide concentration, 6.12×10^{-7} M; excitation slit, 8.0 nm; emission slit, 2.5 nm.

Correlation of Orientation Polarity and Dielectric Constant. Using the data listed in Tables 1 and 2, we constructed a plot of the measured dielectric constant, ε, versus the calculated orientation polarity, f, for the neat and the mixed solvent systems used in this study. The functional dependence be-

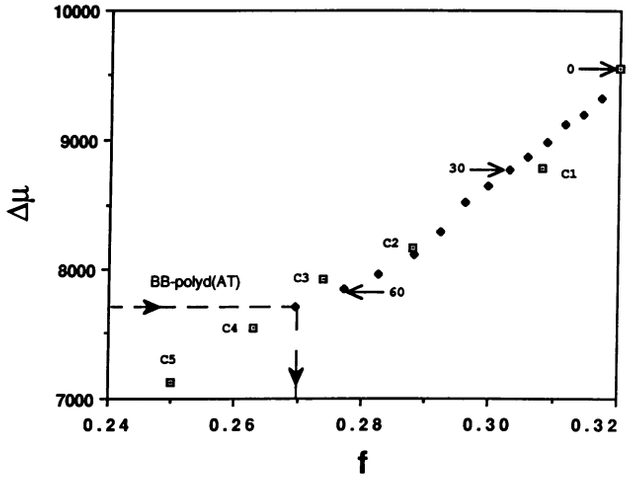

FIG. 3. The relationship between the Stokes' shift, $\Delta\mu$, of bisbenzimide (BB) and the orientation polarity, f, of the solvent in neat organic solvents (□) and mixed solvents (◆). C1–C5 are as defined in Fig. 2. The arrowed path shows the interpolation of an f value from the measured $\Delta\mu$ of the BB–poly[d(AT)]·poly[d(AT)] complex.

tween these two parameters is illustrated in Fig. 4. Later in this paper, we will use this plot to interpolate a dielectric constant value from a given orientation polarity.

The Influence of Ionic Strength on Bisbenzimide Fluorescence. We used NaCl to change the ionic strength of bisbenzimide solutions at constant pH (7.0). For aqueous drug solutions containing between 0 and 6 M NaCl, we measured the difference between the emission and excitation wavelengths. The results of these measurements are presented in Fig. 5 as a plot of the Stokes' shift, $\Delta\mu$, versus the sodium ion concentration. The significant observation is that the Stokes' shift of bisbenzimide is independent of the sodium ion concentration. Consequently, any binding-induced differences in Stokes' shifts that we measure will not artifactually result from ionic strength differences between the bulk solvent and the DNA binding site.

The Influence of pH on the Stokes' Shift. We also evaluated the influence of pH on the fluorescent properties of bisbenzimide in 10 mM phosphate buffer solvents at constant ionic

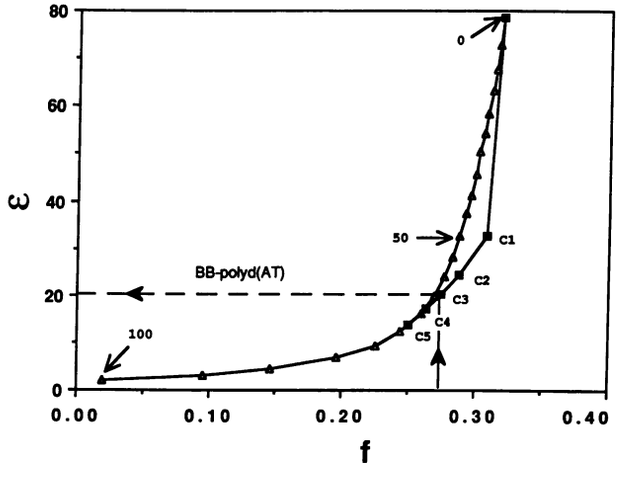

FIG. 4. The correlation between dielectric constant, ε, and the orientation polarity, f, for neat organic solvents (■) and for 1,4-dioxane/water mixed solvents (△). C1–C5 are as defined in Fig. 2. The arrowed path shows the interpolation of an ε value that corresponds to an orientation polarity of 0.27 for the bisbenzimide (BB)–poly[d(AT)]·poly[d(AT)] complex.

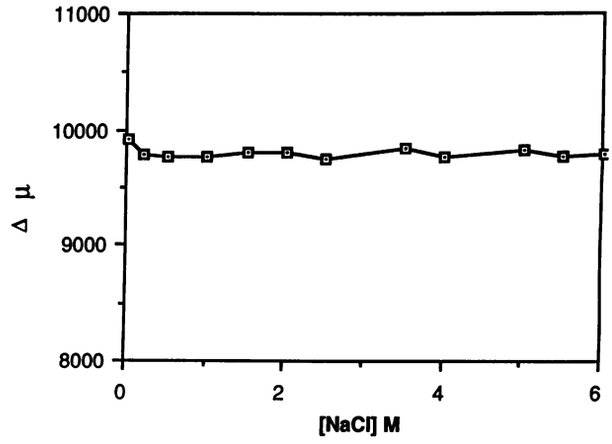

FIG. 5. A plot of the Stokes' shift, $\Delta\mu$, of bisbenzimide at pH 7.0 in 10 mM phosphate buffer versus the concentration of added NaCl.

strength. This control is useful since it is difficult to determine exact pH values for organic/aqueous mixed solvents. Consequently, we measured the excitation and emission spectra of bisbenzimide over the pH range from 3.0 to just under 7.5. We focussed on this pH range since the organic/aqueous mixed solvents used here generally exhibited pH values below neutrality (≈5). [More basic conditions (pH ≥ 8) were avoided since one enters a pK_a region for the drug and the Stokes' shift decreases by ≈650 wavenumbers (data not shown).] Inspection of Fig. 6 reveals that small pH variations in the neat or the mixed solvent systems compared with the pH 7.0 aqueous buffer will not significantly alter the magnitude of the Stokes' shifts we measure for bisbenzimide.

The Influence of DNA Binding on Bisbenzimide Fluorescence. Bisbenzimide binds to A+T-rich tracts of DNA by deep penetration into the minor groove (1, 6–8). The Dickerson group (6) and subsequently Wang in the Rich group (18), have determined crystal structures of complexes in which bisbenzimide is bound to the minor groove of a DNA duplex. To characterize the minor groove microenvironment of the DNA duplex after drug binding, we measured the fluorescence properties of bisbenzimide bound to the poly[d(AT)]·poly[d(AT)] duplex. These fluorescence properties will be sensitive to the local environment within the DNA binding domain. Consequently, bisbenzimide can serve as a reporter molecule or probe of the microenvironment within the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex. It should be emphasized that our measurements characterize the minor groove environment of the

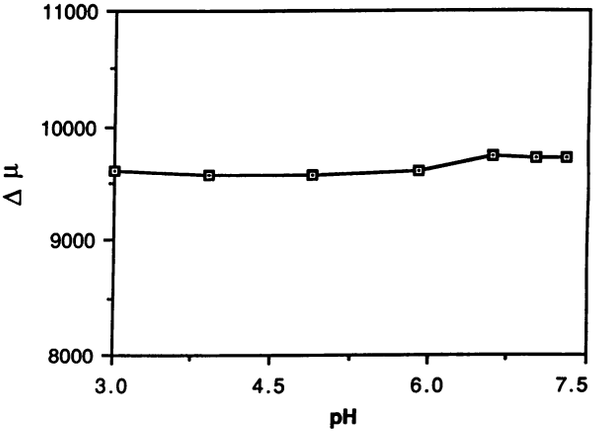

FIG. 6. A plot of the Stokes' shift, $\Delta\mu$, of bisbenzimide in 10 mM phosphate buffer versus the buffer pH.

drug–DNA complex rather than the minor groove of the drug-free duplex.

In Tables 1 and 2, we list the fluorescence properties of bisbenzimide dissolved in the neat and the mixed solvent systems used as reference media in this work. In the last row of each table, we also list the corresponding spectral data we have measured for the fluorescence of bisbenzimide bound to the minor groove of the poly[d(AT)]·poly[d(AT)] duplex. Comparisons between the Stokes' shifts of the free and the duplex-bound bisbenzimide suggest that the minor groove of the poly[d(AT)]·poly[d(AT)]–bisbenzimide complex possesses a microenvironment that is more "organic" than aqueous in nature.

In a previous section, we showed that bisbenzimide in solution exhibits a Stokes' shift that correlates with bulk solvent properties (the orientation polarity) in a manner that is consistent with a general solvent effect. Assuming that this correlation also holds for bisbenzimide bound to DNA, then we can use the Stokes' shift of the DNA-bound ligand as a measure of the local environment within the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex. Inspection of the data in Tables 1 and 2 reveals that the Stokes' shift of minor groove-bound bisbenzimide is similar to that measured in neat propanol or in a dioxane/aqueous mixed solvent containing 60% volume fraction of 1,4-dioxane. Using the plot of Stokes' shift, $\Delta\mu$, versus orientation polarity, f, shown in Fig. 3 (or by inspection of the data in Tables 1 and 2), we interpolate an orientation polarity, f, of $\approx$0.27 for the local environment of the poly[d(AT)]·poly[d(AT)] minor groove in which bisbenzimide is bound. This interpolation is illustrated by the arrowed path in Fig. 3. According to the functional dependence shown in Fig. 4, an orientation polarity, f, of 0.27 interpolates to a dielectric constant, ε, of $\approx$20 D. This interpolation is represented by the arrowed path in Fig. 4. Thus, the environment within the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex is quite nonpolar ($\approx$20 D) compared with the bulk solvent ($\approx$80 D).

We realize that our analysis is approximate since it does not take into account the influence of molecular rigidity and specific bisbenzimide-DNA interactions on the fluorescence properties of the DNA-bound ligand. However, for the reasons noted below, we believe that the latter two effects on the Stokes' shift of bisbenzimide are minor compared with the dominating influence of the orientation polarity of the microenvironment. With regard to molecular rigidity, a comparison between the optical properties of a fluorescent ligand free in solution and covalently linked to a synthetic polymer suggest that molecular rigidity exhibits little, if any, effect on the Stokes' shift of the bound fluorophore (9, 10). With regard to the influence of specific interactions on the Stokes' shift, it should be noted that the neat alcohols and the 1,4-dioxane/aqueous mixed solvents have the potential for different bisbenzimide–solvent interactions. Nevertheless, inspection of the plots in Fig. 3 reveals that the Stokes' shifts of bisbenzimide dissolved in the neat and the mixed solvent systems exhibit similar dependences on the orientation polarity (e.g., the two lines in Fig. 3 are nearly superimposable). This comparison suggests that if specific bisbenzimide–solvent interactions do occur, they do not significantly alter the Stokes' shift. Furthermore, we observe similar Stokes' shifts for bisbenzimide binding to a series of DNA duplexes that possess different functional groups in the minor groove compared with the poly[d(AT)]·poly[d(AT)] duplex (unpublished results). This similarity suggests that if specific bisbenzimide–DNA interactions occur in the minor groove (e.g., hydrogen bonding, van der Waals contacts), they do not significantly alter the Stokes' shift we observe for the free versus the DNA-bound drug. Based on these observations, we conclude that the Stokes' shift we measure for bisbenzimide binding to the poly[d(AT)]·poly[d(AT)] duplex primarily reflects the orientation polarity of the DNA minor groove when complexed with bisbenzimide. Consequently, the analysis in this work provides a reasonable measure of the microenvironment within the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex.

Concluding Remarks. We have compared the fluorescence properties of bisbenzimide in its DNA-bound state with the corresponding properties for bisbenzimide dissolved in neat and organic/aqueous mixed solvents. From this comparison, we conclude that the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex is quite nonpolar and exhibits a local dielectric constant of $\approx$20 D. This result has important implications for our understanding of the molecular forces that dictate and control the binding affinities and specificities of minor-groove-directed DNA binding ligands. For example, the conventional wisdom argues that hydrogen bonding does not provide a significant driving force for association reactions in aqueous solutions, although this view is rapidly changing (11–13). The conventional wisdom is based on the notion that for association reactions in aqueous solution one simply is exchanging solute–solvent hydrogen bonds for solute–solute hydrogen bonds. Consequently, the resulting differential hydrogen bonding effect should be small. We believe that this reasoning is flawed (in particular, for ligand–macromolecule associations) since it implicitly assumes that the local environment within the binding region of the host molecule possesses a dielectric constant similar to that of bulk water (e.g., $\approx$80 D). Our fluorescence data demonstrate that this assumption is not true for the minor groove of the bisbenzimide–poly[d(AT)]·poly[d(AT)] complex, which exhibits a dielectric constant of $\approx$20 D. In such a low dielectric environment, drug–DNA hydrogen bonding interactions are expected to contribute a substantial enthalpic driving force. Calorimetric studies in fact reveal this to be the case (14–17). Thus, when designing DNA binding ligands, one should be cognizant of the fact that ligand–DNA hydrogen bonding interactions within the minor groove may contribute a substantial enthalpic driving force to drug association.

1. Mikhailov, M. V., Zasedatelev, A. S., Krylov, A. S. & Gurskii, G. V. (1981) *Mol. Biol.* **15**, 541–555.
2. CRC (1987/1988) *Handbook of Chemistry and Physics*, ed. Wearst, R. C. (CRC Press, Boca Raton, FL), 68th Ed., pp. E50-E52.
3. Frank, F. & Ives, D. J. G. (1966) *Q. Rev. (London)* **20**, 1–45.
4. Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy* (Plenum, New York), pp. 187–208.
5. Varani, G. & Baldini, G. (1987) *J. Chem. Soc. Faraday Trans. 1* **83**, 1609–1620.
6. Pjura, P. E., Grzeskowiak, K. & Dickerson, R. E. (1987) *J. Mol. Biol.* **197**, 257–271.
7. Harshman, K. & Dervan, P. B. (1985) *Nucleic Acids Res.* **13**, 4825–4835.
8. Zimmer, C. & Wahnert, U. (1986) *Prog. Biophys. Mol. Biol.* **47**, 31–112.
9. Strauss, U. P. & Vesnaver, G. (1975) *J. Phys. Chem.* **79**, 1558–1561.
10. Strauss, U. P. & Vesnaver, G. (1975) *J. Phys. Chem.* **79**, 2426–2429.
11. Turner, D. H., Sugimoto, N., Kierzek, R. & Dreiker, S. D. (1986) *Biochemistry* **25**, 3214–3219.
12. Fersht, A. (1987) *Trends Biochem. Sci.* **12**, 301–304.
13. Freier, S. M., Sugimoto, N., Sinclair, A., Alkema, D., Neilson, T., Kierzek, R., Caruthers, M. H. & Turner, D. H. (1987) *J. Am. Chem. Soc.* **109**, 3783–3785.
14. Marky, L. A. & Breslauer, K. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4359–4363.
15. Marky, L. A., Blumenfeld, K. S. & Breslauer, K. J. (1983) *Nucleic Acids Res.* **11**, 2857–2870.
16. Breslauer, K. J., Chou, W. Y., Ferrante, R., Zaunczkowski, D., Curry, J., Remeta, D. P., Snyder, J. G. & Marky, L. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8922–8926.
17. Breslauer, K. J., Ferrante, R., Curry, J., Zaunczkowski, D., Youngquist, R. S., Dervan, P. B. & Marky, L. A. (1988) in *Structure & Expression, DNA and Its Drug Complexes*, eds. Sarma, M. H. & Sarma, R. H. (Adenine, Guilderland, NY), pp. 273–290.
18. Teng, M. K., Usman, N., Frederick, C. A. & Wang, A. H.-J. (1988) *Nucleic Acids Res.* **16**, 2671–2690.